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X-ray-enhanced cancer cell migration requires the linker of nucleoskeleton and cytoskeleton complex

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The linker of nucleoskeleton and cytoskeleton (LINC) complex is a multifunctional protein complex that is involved in various processes at the nuclear envelope, including nuclear migration, mechanotransduction, chromatin tethering and DNA damage response. We recently showed that a nuclear envelope protein, Sad1 and UNC84 domain protein 1 (SUN1), a component of the LINC complex, has a critical function in cell migration. Although ionizing radiation activates cell migration and invasion in vivo and in vitro, the underlying molecular mechanism remains unknown. Here, we examined the involvement of the LINC complex in radiation-enhanced cell migration and invasion. A sublethal dose of X-ray radiation promoted human breast cancer MDA-MB-231 cell migration and invasion, whereas carbon ion beam radiation suppressed these processes in a dose-dependent manner. Depletion of SUN1 and SUN2 significantly suppressed X-ray-enhanced cell migration and invasion. Moreover, depletion or overexpression of each SUN1 splicing variant revealed that SUN1_888 containing 888 amino acids of SUN1 but not SUN1_916 was required for X-ray-enhanced migration and invasion. In addition, the results suggested that X-ray irradiation affected the expression level of SUN1 splicing variants and a SUN protein binding partner, nesprins. Taken together, our findings supported that the LINC complex contributed to photon-enhanced cell migration and invasion.

KEYWORDS
cell migration, linker of nucleoskeleton and cytoskeleton complex, Sad1 and UNC84 domain protein 1, sublethal dose radiation

1 | INTRODUCTION

The effects of local tumor irradiation on metastasis frequency were first reviewed in 1949.[1] Subsequently, many studies using a variety of cell types, including glioma, hepatocellular carcinoma, pancreatic cancer, breast cancer, lung cancer and noncancerous cells, have demonstrated that photon radiation enhances cell migration and invasion in vivo and in vitro, whereas particle beam radiation suppresses and enhance cell migration and invasion in cell-dependent manner.[2-5] This phenomenon involves various processes, including induction of the epithelial-mesenchymal transition, secretion of proteases such as matrix metalloproteinases, and activation of cell signaling pathways.[6-8] However, the underlying molecular mechanisms remain poorly understood.

The Sad1-UNC-84 (SUN) homology domain proteins of the inner nuclear membrane (INM) are widely conserved in all eukaryotes and

share a common C-terminal motif of approximately 200 amino acids, termed SUN domain. SUN proteins and the outer nuclear membrane (ONM)-spanning nesprin proteins interact with each other in the perinuclear space via their luminal domain, SUN and KASH (Klarsichet/Anc1/Syne1 homology), respectively, to form a multifunctional nuclear membrane protein assembly called the linker of nucleoskeleton and cytoskeleton (LINC) complex.[9-11] SUN proteins interact with the nucleoskeleton, including chromatin and nuclear lamina. Nesprins interact with the cytoskeleton, including actin filaments, intermediate filaments and microtubule motors; therefore, the LINC complex directly connects the nuclear interior to cytoskeleton. LINC complex and nuclear lamins form a solid scaffold for diverse functions, including nuclear migration,[12] nuclear shaping and positioning,[13,14] maintaining the centrosome-nucleus connection,[15,16] mechanotransduction,[17,18] DNA repair,[19,20] nuclear membrane spacing,[21] cell migration,[16,22-25] and moving chromosomes within the nucleus during meiosis.[26]

Somatic mammalian cells express SUN1 and SUN2, both of which are required for cell migration.[27,28] The human *SUN1* gene contains 22 exons and generates more than 10 splicing variants that are distinguished by variable deletions just upstream from the transmembrane domain, between exons 6 and 9.[28] The largest human SUN1 splicing variant is composed of 916 amino acids (aa; EAW87177) and is referred to as SUN1_916. In addition to this variant, there are other variants, including SUN1_785 and SUN1_888 (AB648918), which encode 785 and 888 aa, respectively.[28] We previously showed that SUN1 splicing variants play crucial functions in cell migration.[28]

Therefore, in this study, we evaluated whether SUN1 proteins are involved in photon-enhanced cell migration and invasion.

2 | MATERIALS AND METHODS

2.1 | Cell culture, western blotting, antibodies and plasmids

Human breast cancer MDA-MB-231 cells were maintained in DMEM (Wako Pure Chemical, Osaka, Japan) supplemented with 10% (w/v) FBS. Western blotting was performed as previously described.[29] Anti-SUN1, anti-nesprin-1 and anti-β-actin antibodies were purchased from Sigma Aldrich. Anti-SUN2 antibody was from Millipore. Plasmids for the expression of GFP or GFP-tagged SUN1_916 (GFP-SUN1_916) were described previously.[28]

2.2 | Colony formation assay and clonogenic survival curves

Immediately after irradiation, cells were harvested with trypsin-EDTA, and seeded into culture dishes. Fourteen days after culturing, cells were fixed with 10% formalin, and stained with 0.04% crystal violet solution. Then, more than 50 cells in each colony were counted as surviving cells. Surviving fractions (SF) against physical doses were plotted and fitted to surviving curves using the following linear-quadratic model as previously reported:[4] $SF = \exp(-\alpha \times D - \beta \times D^2)$. D indicates radiation dose.

2.3 | Radiation

For X-ray radiation, medium was replaced with warmed serum-free medium just before irradiation. Cells were irradiated with 1.0 Gy/min of 4 MV X-ray radiation from a linear accelerator (EXL-6SP; Varian, Palo Alto, CA, USA) at Osaka University. After irradiation, the medium was immediately changed to fresh culture medium with 10% FBS. Carbon ion beam irradiation was performed at the Heavy Ion Medical Accelerator in Chiba (HIMAC), National institute of Radiological Sciences, Japan. The energy and the dose rate were 290 MeV/nucleon and 3.3 Gy/min, respectively. The cells were irradiated at the center of a 6-cm spread-out Bragg peak.

2.4 | siRNA-mediated knockdown

Sequences for the siRNA pools against SUN1 and SUN2 were described previously[28,29] and obtained from Nippon Gene, Japan. siRNA against SUN1_888 and SUN1_916-specific regions were designed by and obtained from Nippon Gene and the activities were previously confirmed.[28] Cells were transfected with targeting siRNA or a non-targeting siRNA pool (Thermo Fisher, MA, USA) using Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA, USA).

2.5 | RT-PCR

Total RNA was isolated using a PureLink RNA Mini Kit (Life Technologies). PCR amplification was performed using Emerald Amp PCR (Takara Bio) and primers for total SUN1 was indicated previously.[28] SUN1_916-specific primers were as follows: 5′-GAATCAAAAGCTCATGCCAGTT-3′ and 5′-TCTGCAGCAAGAAGTAACCTG-3′.

2.6 | Cell migration

Cell migration assays were performed as previously described using a 48-well microchemotaxis Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) and polycarbonate filter with 8-μm pores (Neuro Probe).[30] The lower side of the filter was precoated with 10 μg/mL collagen type I-C (Nitta Gelatin, Osaka, Japan). After incubation for 3 hours, the number of cells that had migrated to the lower side was counted in 3 independent fields. These experiments were repeated a minimum of 4 times.

2.7 | Matrigel invasion assay

Chemotaxicell filters with 8-μm pores (Kurabo Industries, Osaka, Japan) were precoated with 100 μL of 0.1 μg/mL Matrigel (Becton Dickinson Bioscience, Franklin Lakes, NJ, USA). After incubation for 24 hours, the number of cells that had invaded to the lower side

through the pores was counted in 3 independent fields. In both assays, FBS was used as a chemoattractant. These experiments were repeated a minimum of 4 times.

2.8 | Statistics

All experiments were performed at least 3 times, and the results were expressed as mean values with standard deviations. Statistical significance was evaluated using 2-sided Student's *t* tests. Differences with *P*-values of <.05 were considered statistically significant.

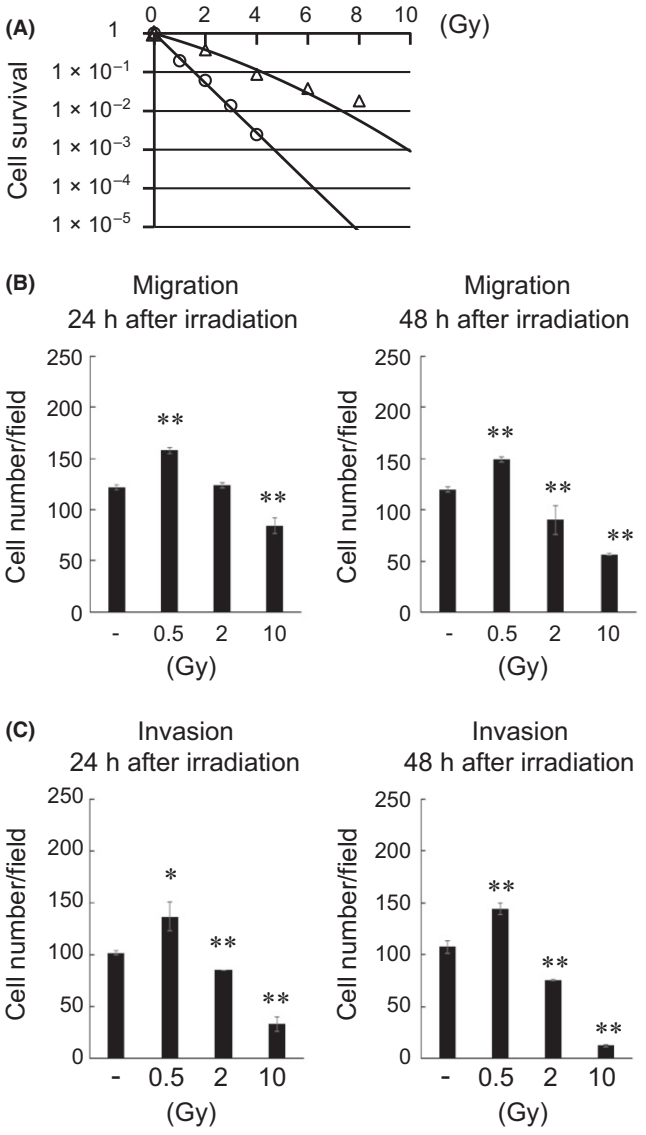

FIGURE 1 Low doses of X-ray radiation promoted cell migration and invasion. A, Clonogenic survival curves after photon irradiation. MDA-MB-231 cells were exposed to X-ray (open triangles) or carbon ion beam (open circles) radiation, and colony formation assays were performed. The approximate 80% survival dose was used as the sublethal dose. B, Cells were exposed to the indicated dose of X-ray radiation, and migration activities were examined 24 and 48 h later. Each bar represents the mean ± SD. *$P < .05$, **$P < .01$. C, Cells were exposed to the indicated dose of X-ray radiation, and invasion activities were examined 24 and 48 h later. Each bar represents the mean ± SD. *$P < .05$, **$P < .01$

3 | RESULTS

3.1 | Sublethal doses of X-ray but not carbon ion beam radiation activated breast cancer cell migration and invasion

To examine the involvement of SUN proteins in photon-enhanced migration and invasion, we used MDA-MB-231 cells, which are highly invasive human breast cancer cells. To obtain the biologically equivalent doses for X-ray and carbon ion beam, we examined the clonogenic survival using the colony formation assay and 0.5 Gy X-ray radiation was decided as the sublethal dose of X-ray radiation for MDA-MB-231 cells (Figure 1A). Cells were irradiated with the indicated dose of X-ray, and then 24 or 48 hours later invasion or migration activities were measured. As expected, 10.0 Gy X-ray radiation suppressed cell migration after 24 hours; however, 0.5 Gy

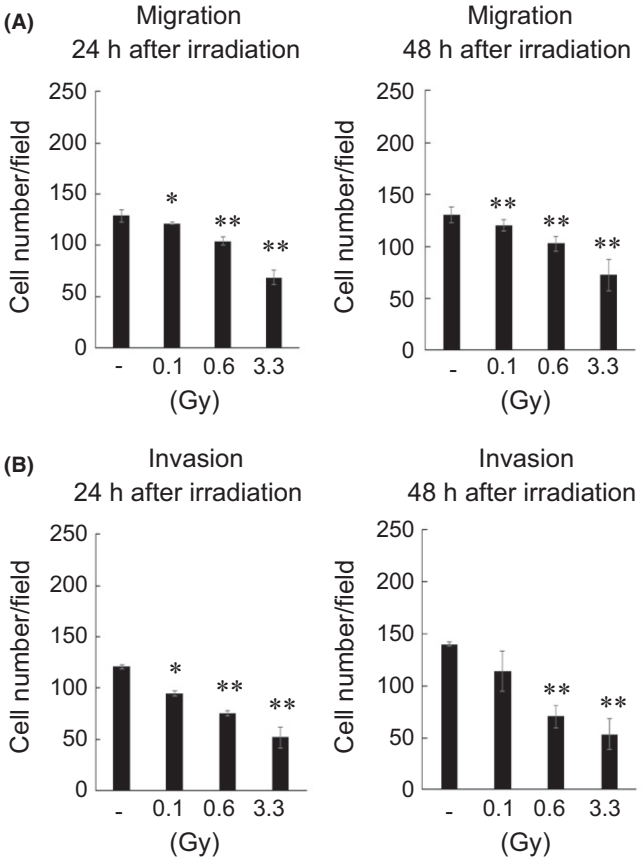

FIGURE 2 Carbon ion beam radiation suppressed cell migration and invasion in a dose-dependent manner. A, Cells were exposed to the indicated doses of carbon ion beam radiation, and migration activities were examined 24 or 48 h later. Each bar represents the mean ± SD. *$P < .05$, **$P < .01$. B, Cells were exposed to the indicated dose of carbon ion beam radiation, and invasion activities were examined 24 and 48 h later. Each bar represents the mean ± SD. *$P < .05$, **$P < .01$

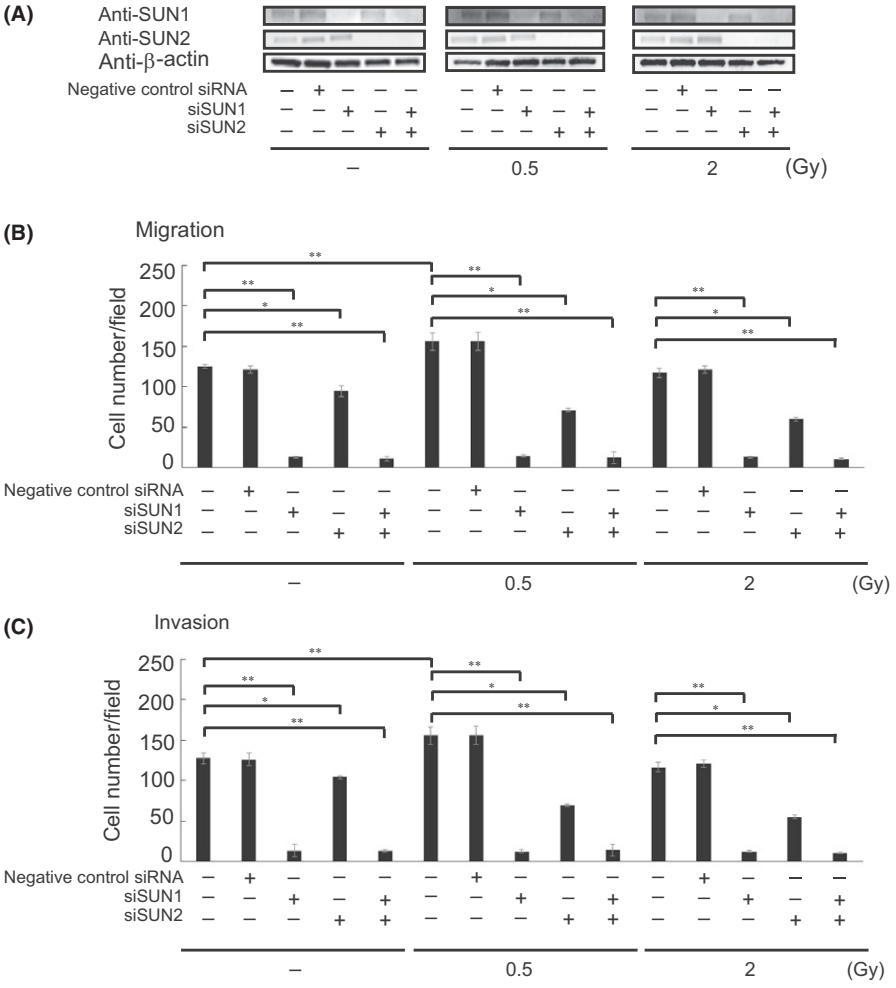

FIGURE 3 X-ray-enhanced migration and invasion required SUN1. A, Cells were transfected with siRNA targeting SUN1 (siSUN1) and/or SUN2 (siSUN2) and incubated for 48 h. The cells were then exposed to X-ray radiation and after 24 h incubation SUN1 and SUN2 expression levels were analyzed by western blotting. B and C, Cells were transfected with siRNA targeting SUN1 (siSUN1) and/or SUN2 (siSUN2) and incubated for 48 h. Cells were exposed to X-ray radiation and incubated for 24 h. Then, invasion activities were measured. Each bar represents the mean ± SD. *$P < .05$, **$P < .01$

radiation promoted migration (Figure 1B). This effect was also observed 48 hours after radiation; that is, 0.5 Gy radiation promoted cell migration, whereas 10.0 Gy radiation suppressed migration (Figure 1B). Note that there are no significant differences in migration activity between non-radiated and 2.0 Gy X-ray radiated cells after 24 hours exposure, whereas 2.0 Gy radiation slightly suppressed it after 48 hours, reproducibly. Cell invasion was also promoted after exposure to a subleathal dose (ie, 0.5 Gy but not 2.0 or 10.0 Gy X-ray radiation both 24 and 48 hours after radiation; Figure 1C).

Next, to assess the effects of carbon ion beam radiation on migration and invasion, cells were irradiated with 0.1, 0.6, and 3.3 Gy of carbon ion beam; these doses resulted in the same clonogenic survival as observed for 0.5, 2.0 and 10.0 Gy X-ray radiation, respectively (Figure 1A). In contrast to X-ray radiation, carbon ion beam radiation suppressed both migration and invasion in a dose-dependent manner 24 and 48 hours after radiation (Figure 2A,B).

These data demonstrated that sublethal doses of X-ray but not carbon ion beam radiation-enhanced migration and invasion. Therefore, we have investigated the underlying molecular mechanism of the X-ray-enhanced cancer cell migration and invasion. In addition, because X-ray-enhanced cancer cell migration was observed both 24 and 48 hours after radiation, cells were incubated for 24 hours after radiation hereafter.

3.2 | Nuclear envelope proteins SUN1 and SUN2 were required for X-ray-enhanced migration and invasion

SUN1 and SUN2 are known to be involved in cell migration.[27,28] Thus, to examine the involvement of SUN proteins in X-ray-enhanced migration and invasion, SUN1 and/or SUN2 were depleted using siRNA for 48 hours, and the cells were irradiated with X-ray radiation. There are no significant differences in SUN1 and SUN2 protein expression levels after 0.5 and 2.0 Gy X-ray irradiation (Figures 3A and S1). Depletion of SUN1 and SUN2 was confirmed by western blotting (Figure 3A). Both 0.5 and 2.0 Gy X-ray radiation did not affect the SUN1 and/or SUN2 protein depletion by siRNA (Figure 3A, middle and right). Note that carbon ion beam irradiation also did not affect the protein expression levels of SUN1 and SUN2 (Figure S1, discussed below).

Without X-ray radiation, depletion of SUN1 alone or both SUN1 and SUN2 reduced migration and invasion to <10% compared with

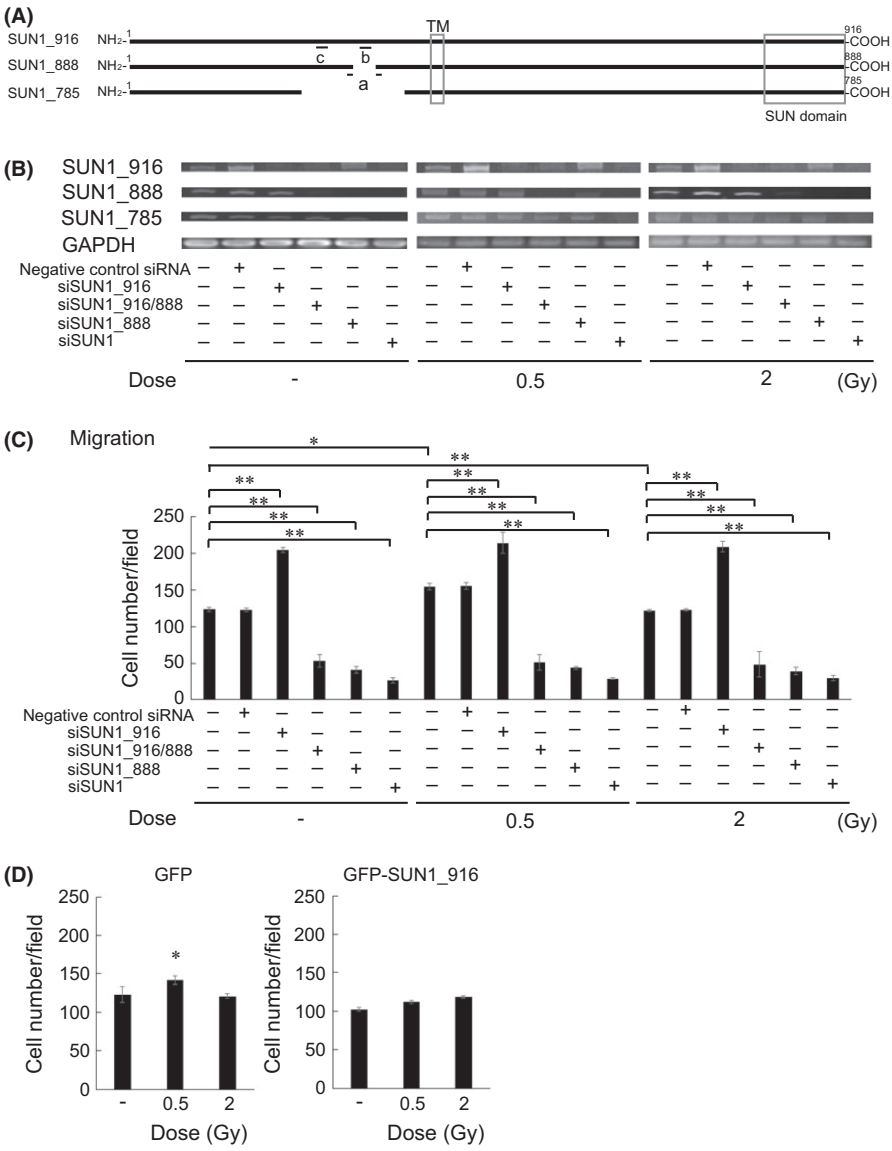

FIGURE 4 SUN1 variants had distinct functions in low-dose X-ray-enhanced cell migration and invasion. A, Schematic presentation of SUN1 splicing variants and siRNA specifically targeting SUN1_888 (a, siSUN1_888), SUN1_916 (b, siSUN1_916), and both SUN1_916 and SUN1_888 (c, siSUN1_916/888). B and C, Indicated siRNA were transfected into cells for 48 h. The cells were then exposed to X-ray radiation and incubated for 24 h. Depletion of SUN1 variants was confirmed by PCR, and migration activity was measured at 24 h after X-ray radiation. Each bar represents the mean ± SD. $*P < .05$, $**P < .01$. D, Cells were transfected with GFP or GFP-SUN1_916. After incubation for 24 h, cells were exposed to the indicated dose of X-ray. Twenty-four hours later, cell migration activities were analyzed. $*P < .05$

control or negative control siRNA-transfected cells, whereas SUN2 depletion reduced migration and invasion to approximately 80% (Figure 3B,C). In addition, 0.5 Gy but not 2.0 Gy X-ray radiation promoted the migration and invasion of nontransfected or negative control siRNA-transfected cells (approximately 130% compared with nonirradiated cells); however, the same dose of X-ray radiation did not promote migration or invasion in SUN1-depleted and/or SUN2-depleted cells (Figure 3B,C, left). Therefore, these data demonstrate that both SUN1 and SUN2 were required for photon-enhanced migration and invasion.

3.3 | SUN1 splicing variants had distinct functions in photon-enhanced cell migration and invasion

The mammalian *SUN1* gene generates various splicing variants, including SUN1_785, SUN1_888 and SUN1_916.[28,31] SUN1_916 is the most predominant and detectable variant in western blotting (Figure 3A). We recently showed that overexpression of SUN1_916 inhibits cell migration, whereas SUN1_785 and SUN1_888 promote cell migration.[28] Thus, to investigate the involvement of SUN1 splicing variant(s) in X-ray-enhanced cell migration and invasion, cells were transfected with variant-specific siRNA for 48 hours to specifically downregulate SUN1_916 and/or SUN1_888 (Figure 4A). The cells were then exposed to X-ray radiation and incubated for 24 hours. Depletion of SUN1 splicing variants was confirmed by PCR (Figure 4B) because SUN1_888 and SUN1_785 could not be detected at the protein level due to low expression.[28] Cells transfected with negative control siRNA showed photon-enhanced migration and invasion 24 hours after irradiation, consistently with Figure 1 (see 0.5 Gy dose X-ray irradiated cells in Figures 4C, middle, and S2, middle). Depletion of SUN1_916 activated migration and invasion without X-ray radiation, and this effect was maintained but not further increased after exposure to 0.5 Gy X-ray radiation (Figures 4C, middle, and S2, middle, siSUN1_916 transfected cells). In contrast, depletion of SUN1_888 alone or depletion of both SUN1_916 and SUN1_888 decreased migration and invasion and

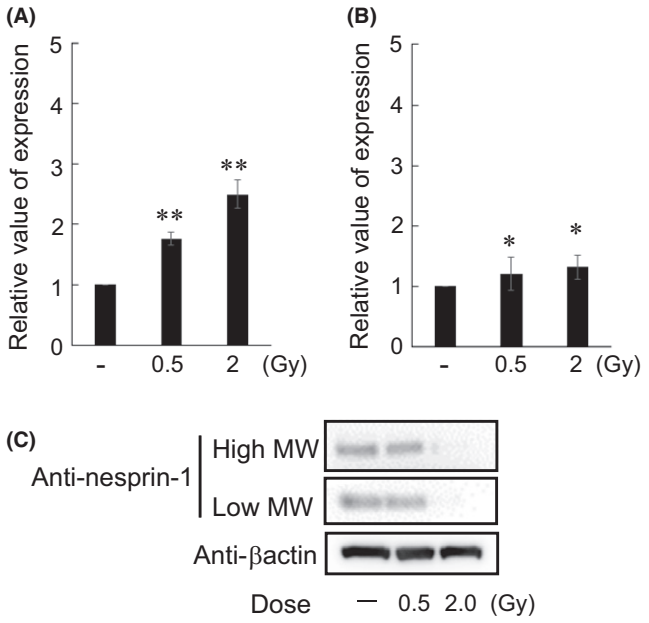

FIGURE 5 Expression of linker of nucleoskeleton and cytoskeleton (LINC) complex components was altered after X-ray radiation. A and B, Cells were exposed to the indicated dose of X-ray radiation. Twenty-four hours later, mRNA were collected, and real-time PCR was performed using primers to detect all SUN1 variants (A) or SUN1_916 (B) $^{*}P < .01$, $^{**}P < .05$. C, Cells were exposed to the indicated dose of X-ray radiation. Twenty-four hours later, the expression of nesprin-1 was examined by western blotting

also suppressed photon-induced migration and invasion (Figures 4C and S2). This result indicates that both SUN1_888 and SUN1_916 might be required for photon-enhanced cell migration. However, overexpression of SUN1_916 suppressed both radiation-independent cell migration and photon-enhanced cell migration (Figure 4D). Therefore, we assumed that SUN1_888 was required for photon-enhanced migration and invasion; however, SUN1_916 suppressed migration and invasion at steady state, whereas depletion of SUN1_916 stimulated migration and invasion to a greater extent than photon-enhanced cell migration. Note that it was not possible to design siRNA specifically targeting SUN1_785 because the SUN1_785 sequence mostly overlaps those of SUN1_916 and SUN1_888 (Figure 4A).

3.4 | X-ray radiation altered the expression of linker of nucleoskeleton and cytoskeleton complex components

We then measured the expression of *SUN1* mRNA by real-time PCR. Interestingly, X-ray radiation induced an increase in *SUN1* mRNA, including all SUN1 variants (Figure 5A). However, X-ray irradiation did not increase the amount of SUN1_916 mRNA expression (Figure 5B), suggesting that X-ray radiation increased the expression of *SUN1* variants other than SUN1_916 (eg, SUN1_785 or SUN1_888). We could not quantify other SUN1 splicing variants except SUN1_916 because it was impossible to design primers specifically to detect other SUN1 variants, such as SUN1_888 or SUN1_785, due to the overlap of the coding region with SUN1_916 (Figure 4A).

After 2.0 Gy X-ray radiation, although total *SUN1* mRNA expression (and likely SUN1_785 and/or SUN1_888 expression) was increased (Figure 5A), migration and invasion were suppressed. To explain this phenomenon, the expression of SUN1 binding partners, nesprin-1 and nesprin-2, which bind to both SUN1 and the cytoskeleton, was investigated. Interestingly, the expression levels of nesprin-1 and nesprin-2 were not affected by 0.5 Gy X-ray radiation but were dramatically decreased after exposure to 2.0 Gy X-ray radiation (Figures 5C and S3), suggesting that 2.0 Gy of X-ray radiation suppressed migration and invasion through disrupted function of LINC complex, which is composed of SUN and nesprin proteins. Thus, these results supported that exposure to 2.0 Gy radiation suppressed migration and invasion.

4 | DISCUSSION

In this study, we showed that a sublethal dose of X-ray radiation promoted cell migration in MDA-MB-231 breast cancer cells, whereas carbon ion beam radiation suppressed cell migration in a dose-dependent manner. Intensity modulated radiation therapy has been applied in breast cancer treatment;[32,33] however, the irradiation delivers a low dose of X-rays to the periphery of the target tumor because of the nature of multifield radiotherapy.[34] Therefore, it is important to determine the molecular mechanisms underlying the low dose effects of ionizing radiation on migration and invasion.

Linker of nucleoskeleton and cytoskeleton complex components, both SUN1 and SUN2 proteins, were found to be essential for photon-enhanced migration and invasion. We previously found that one of the SUN1 splicing variants, SUN1_916, inhibits cell migration, whereas other splicing variants, SUN1_785 and SUN1_888, promote cell migration using several human cancerous cells, HeLa, MDA-MB-231 and HT1080 cells.[28] We showed that SUN1_888 was involved in migration induced by a sublethal dose of X-ray radiation, whereas SUN1_916 suppressed photon-enhanced cell migration. In addition, depletion of SUN2 in combination with a sublethal dose of radiation intriguingly reduced tumor cell migration and invasion, suggesting that SUN2 is also required for the X-ray-enhanced cell migration (Figure 3B,C, middle). SUN2 is known to accumulate along dorsal actin cables to form transmembrane actin associated nuclear (TAN) lines,[22] which are anchored to the nuclear lamina through SUN2 interaction with A-type lamins and play a crucial role in nuclear migration.[35] Moreover, we showed that after exposure to 2.0 Gy X-ray radiation, nesprin-1 and nesprin-2 expression levels were dramatically reduced. Thus, our results suggested that X-ray radiation induced the switching of LINC complex components and contributed to stimulation and suppression of cell migration and invasion. In addition, we showed that suppression of SUN1_888 expression abrogated the migration of both nonirradiated and irradiated cells, although the expression levels of *SUN1_785* and *SUN1_888* mRNA could not be evaluated due to the genetic structure of each variant.

These results provided important insights into the effects of radiation on cancer cell migration and invasion and may facilitate the development of novel approaches for the treatment of cancer, whereas further studies should be performed using other various cancerous and noncancerous cells.

Various studies have shown that ionizing radiation not only can kill tumor cells but also promote cell migration and invasion via various mechanisms, including activation of the epithelial mesenchymal transition (EMT),[8] and activation of Rho protein families[6] and integrins.[36] Because the LINC complex is composed of INM and ONM localized proteins (ie, SUNs and nesprins) and interacts with both nuclear skeleton and cytoskeleton, the LINC complex might be an upstream regulator for the above diverse mechanisms via EMT transition and cytoskeleton-related functions during the activation of Rho or integrins. Note that we have observed that expression of LINC complex components affected the expression level of E-cadherin (H. Imaizumi, M. Hieda, unpublished data).

The sublethal X-ray enhances cell migration, whereas the carbon ion beam radiation suppressed it. Currently we have no data on whether LINC complex is involved in the suppressed cell migration by carbon ion beam radiation and we did not observe the obvious alteration of LINC complex components after carbon ion beam radiation in MDA-MB-231 cells (Figure S1) and in A549 lung cancer cells.[37] Therefore, different regulations might exist in suppression of cell migration after carbon ion beam radiation.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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